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A Critical Review of Analytical Ultracentrifugation and Field Flow Fractionation Methods for Measuring Protein Aggregation

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ABSTRACT

Analytical ultracentrifugation (AUC) and field flow fractionation (FFF) are 2 important biophysical methods for measuring protein aggregates. Both methods can separate protein monomer from its aggregate forms under a broad range of solution conditions. Recent advances in instrumentation and data analysis, particularly in the field of analytical ultracentrifugation technology, have significantly improved the capability and sensitivity of these biophysical methods for detecting protein aggregates. These advances have resulted in an increased use of these methods in the biopharmaceutical industry for characterization of therapeutic proteins. However, despite their many advantages over conventional methods, the difficulty in the use of the instrumentation and the complexity of data analysis process, have often hampered the widespread use and proper interpretation of data. This article reviews the recent progress in both technologies, and a few case studies are also presented to discuss their advantages and limitations.

KEYWORDS: Analytical ultracentrifuge, sedimentation velocity, SEDFIT, field flow fractionation, protein aggregates

INTRODUCTION

Protein aggregation is a major concern for biopharmaceutical products because it can potentially affect drug activity, immunogenicity, and pharmacokinetic and pharmacodynamic profiles.[1-4] Protein aggregates are common degradation products encountered during different stages of manufacturing, formulation, and storage. It is important to detect, control, and minimize the aggregates in the final products to ensure their safety and efficacy.

Protein aggregation can be measured by different biophysical methods. Size exclusion chromatography (SEC) is one of the most commonly used methods for detecting and characterizing protein aggregates in the biopharmaceutical industry. The method is sensitive, reproducible, and relatively easy to use. It is also a high throughput method and can be fully validated. However, SEC has several major limitations. First, it only operates over limited buffer conditions. Very often it requires the addition of a high concentration of salt or organic solvent to eliminate nonspecific interactions between the protein and the column matrix. This requirement can sometimes generate artificial peaks in the chromatogram that may not be present in the original drug substance and product. Second, there is a significant dilution during the chromatography process, so an aggregate formed by weak reversible interaction can be dissociated as the concentration decreases. Last, SEC separates aggregates over a limited size range and has poor resolution for larger soluble aggregates, which may be eluted out at the void volume. Insoluble aggregates are often filtered out by the column matrix and, therefore, are never seen by the detectors.

In contrast, both analytical ultracentrifugation (AUC) and field flow fractionation (FFF) methods provide useful alternatives for aggregate characterization.[5,6] Both methods cover a wide range of molecular sizes; for instance, the FFF method can resolve the aggregates or particles from 0.001 to 50 μm in size.[7] There are no matrices involved that may influence the separation. The methods can be applied to protein under a broad range of buffer conditions, even in the formulation buffers. In addition, both methods have a rigorous theoretical basis and, therefore, can provide an accurate estimation about quantity, size, and shape of multiple protein aggregate species without the use of molecular weight standards.[8-11] Despite these advantages, the precision and accuracy of these methods for detecting low levels of protein aggregates are not well established, and it requires highly trained personnel for instrumentation operation and data analysis. In this study, we have reviewed the use of both AUC and FFF technologies for quantitative detection of protein aggregates. Several case study examples are discussed. Considerations for improved experimental condition and data analysis are also proposed to improve quantitation of protein aggregates.

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Materials and Methods

Materials

Two humanized IgG1 monoclonal antibodies (mAb) used in our investigations were produced by Genentech Inc (South San Francisco, CA). Both samples were expressed in Chinese hamster ovary (CHO) cells and were purified with the standard chromatography process, which includes protein A and ion-exchange chromatography steps. The materials were formulated into different buffers and excipients using tangential flow filtration, and then aseptically filled into 5-mL glass vials. The degraded samples were generated by incubating the vials at elevated temperature of 30ºC or 40ºC for a different time period.

Sedimentation Velocity

Sedimentation velocity experiments were conducted in a Beckman XLI ultracentrifuge (Beckman Coulter Inc, Palo Alto, CA). The experiments were performed at 40 000 and 60 000 rpm at 20°C using the charcoal-filled Epon 12-mm double-sector centerpieces. The moving boundary was monitored by repetitive radial scanning at a constant step size of 0.003 cm at 280 nm using a UV absorption optical system.

Sedimentation velocity data were analyzed and simulation data were created using the software program SEDFIT (National Institutes of Health, Bethesda, MD) to generate the sedimentation coefficient distribution of protein samples.[12] The model for the simulation data contained noninteracting antibody monomer and dimer species with the assigned random noises at different levels.

Size Exclusion Chromatography

Size exclusion chromatography experiments were performed on an Agilent 1090 high-performance liquid chromatography (HPLC) system with a diode-array detector (Agilent Technologies, Inc., Palo Alto, CA) and a multiangle light scattering detector, DAWN (Wyatt Technology, Santa Barbara, CA). Approximately 20 μg of sample was loaded on a TSK SUPER SW 3000 column (4.6 × 300 mm) (Tosoh Bioscience LLC, Montgomeryville, PA) and eluted at 0.2 mL/min in an isocratic mode using a mobile phase containing 0.1 M potassium phosphate and 0.1 M sodium chloride at pH 6.8. The UV data were collected at 280 nm and analyzed by HP Chemstation software (Agilent Technologies, Inc., Palo Alto, CA).

Field Flow Fractionation

Field flow fractionation experiments were conducted on an AF 2000 Avalanche system (Postnova Analytics Inc, Salt Lake City, Utah). This device contains 2 isocratic HPLC pumps to control the focusing flow and tip flow. The cross-flow is controlled by a Kloehn dual syringe pump system. The regenerated cellulose membrane with 10 kd molecular weight cutoff and low protein binding was used. The sample was injected at 10 μL and the cross-flow was set at 6 or 3 mL/min for fragments, intact monomer, and smaller aggregates, and 0.5 mL/min for larger aggregates and particulates. The sample was eluted in phosphate-buffered saline (PBS) (9 mM phosphate buffer, 137 mM NaCl at pH 7.2). The elution was monitored at 280 nm by an Agilent UV diode-array detector.

Results and Discussion

Analytical Ultracentrifugation

Background

The analytical ultracentrifuge is an extremely versatile tool and has been used extensively to study the solution structure and conformation of macromolecules.[5] The instrument essentially consists of a high-speed centrifuge, a rotor with several cell compartments, and the optical systems that can measure the concentration gradients of proteins under centrifugal force. The protein samples are loaded into a centerpiece that is covered by a quartz or sapphire window on each side. The sedimentation of proteins under a centrifugal field along the radial position can be determined at any time by either absorption or interference optical systems. The study of concentration distributions under a centrifugal field can yield the structural, conformation, and molecular interaction information, such as molecular weight, sedimentation coefficient, diffusion coefficient, binding affinity, and virial coefficient.

The analytical ultracentrifuge operates in 2 basic modes: sedimentation velocity and sedimentation equilibrium. The sedimentation velocity experiment is usually conducted at relatively high rotor speeds, and it only takes a few hours to complete for most of large protein molecules. Each protein species, based on its molecular mass and shape, can form a unique boundary and sediment at a characteristic speed. The velocity and shape of the moving boundary is used to estimate the sedimentation coefficient, diffusion coefficient, molecular weight, and even equilibrium constants of interacting species.[11,12] The sedimentation equilibrium experiment is often operated at relatively lower rotor speed. The sedimentation of proteins under the lower centrifugal field is opposed by the diffusion, and eventually when they reach equilibrium, a time invariant exponential concentration gradient of protein is established throughout the centrifuge cell. The concentration gradient at equilibrium can be rigorously described by a thermodynamic theory and has been widely used to determine the mol ecular weight, stoichiometry, binding affinity, and virial coefficient of interacting or self-associating proteins.[13]

With recent advances in data analysis software, particularly for sedimentation velocity analysis, AUC has become an increasingly important biophysical method for detecting and characterizing the size distribution of therapeutic proteins and their aggregates in the biopharmaceutical industry. Sedimentation velocity is a preferred method for analyzing protein size distribution because it separates each noninteracting species with a unique boundary. For a fast reversible interacting system, the boundary formed during sedimentation process will become a reaction boundary that corresponds to the individual interacting species, as well as to the equilibrium between these species. Analysis of the amplitude and asymptotic shape of the reaction boundary can yield the thermodynamic information of the interacting systems.[14-16] In a manner similar to SEC chromatograms, the sedimentation coefficient distribution of each species can be plotted as a peak and easily integrated for quantitation. Several sedimentation velocity analysis methods have been developed over the years. The integral sedimentation coefficient distribution G(s) was originally introduced by Van Holde and Weischet.[17] This method is based on a geometric division of the sedimentation boundaries that were extrapolated to infinite time to eliminate the effect of diffusion during sedimentation. The extrapolated s-values for each boundary fraction produce an integral sedimentation coefficient distribution G(s).[13] The method is model independent and provides very useful and rapid diagnostic information about the heterogeneous distribution of a sample, its association behavior, and hydrodynamic or thermodynamic nonideality. The time derivative or dc/dt method was originally developed by Walter Stafford.[18] In this approach, a group of scans taken at closely spaced time intervals are subtracted in pairs and then transformed to the distribution of apparent sedimentation coefficient distribution, g(s*), with the peaks that look like a chromatogram. This method has been very useful, since the time-invariant noise in the data was removed algebraically by pair-wise subtraction. The method is model independent and does not rely on any assumptions about the structure and conformation of the protein. However, it is difficult to resolve all species because there is no correction for diffusion, and the number of scans used for analyses is limited. The c(s) analysis is a more recent method that was developed by Peter Schuck in his program SEDFIT.[12] This method uses finite-element solutions of the Lamm equation by directly fitting the velocity data. The sedimentation coefficient distribution from this method has a much improved resolution and covers a broader size distribution than the g(s*) distribution, because it explicitly corrects the broadening from diffusion by the numerical methods and all of the scans can be used in the analysis. In addition, this method includes a sophisticated regularization routine to remove the artificial peaks caused by the noise in raw data. This method has been increasingly used in the biopharmaceutical industry for monitoring the size distribution of aggregates. The genetic algorithms method by Borris Demeler represents further progress for sedimentation velocity data analysis.[19] The method uses an evolutionary program as a stochastic optimization operation to obtain both sedimentation and diffusion coefficients. It is model independent and provides high resolution for these parameters. However, the method requires heavy computation and can take substantial time with a regular computer.

Figure 1 shows the size distribution of a degraded monoclonal antibody sample using SEC-UV, on-line light scattering, and sedimentation velocity methods. The sedimentation velocity data were analyzed using SEDFIT to yield a sedimentation coefficient distribution of protein. The major degradation products, including 2 fragment peaks and several aggregate peaks were resolved by both the SEC and sedimentation velocity methods. However, the sedimentation velocity method clearly shows the better separation of

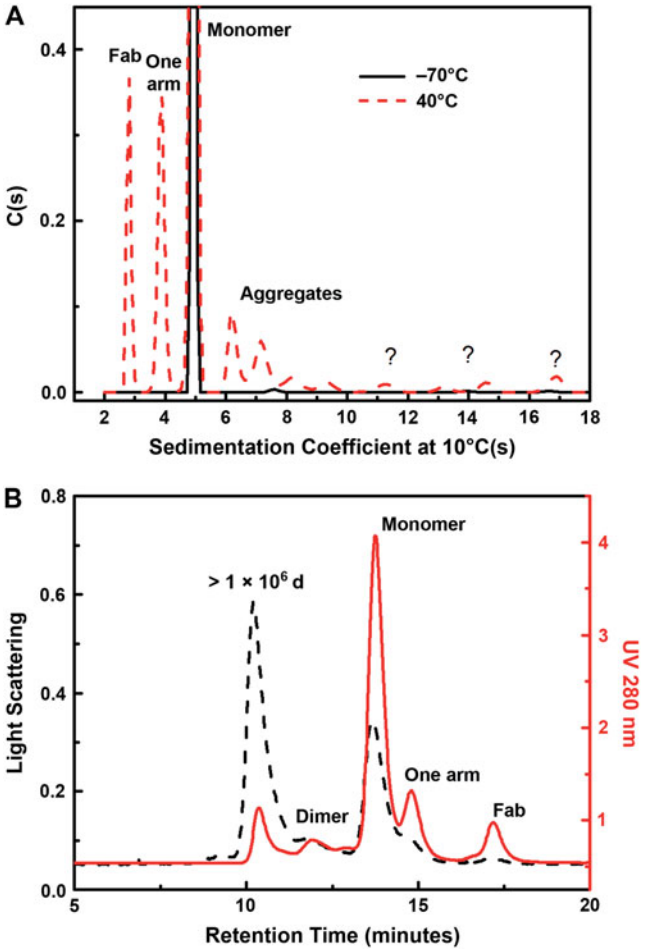

Figure 1. Sedimentation velocity experiment (A) of an mAb upon stored at −70ºC (—) and 40ºC (---) for 6 months. The degraded mAb stored at 40ºC was also analyzed by size exclusion chromatography (SEC) (B). The peaks eluted from SEC were monitored by UV (—) and an on-line multi-angle light scattering detector (---).

fragments and aggregates than the SEC method. In addition, more aggregate peaks were resolved by the sedimentation velocity method. Although, the identities of those additional peaks were not fully understood, and additional characterization work would be required to substantiate these peaks, the data clearly demonstrate that sedimentation velocity method can be a very powerful method to monitor the size distribution of aggregates.

Instrumentation Conditions

The analytical ultracentrifuge measures protein concentration at a specific radial position and time point during the sedimentation process. The precision and accuracy of these measurements are essential for detection, particularly for measuring trace amounts of soluble aggregates.

For a UV absorption optical system, the radial position is measured by a moving slit/lens assembly above the photomultiplier tube located on the bottom of the chamber. The instrument calibrates its radial position using the inner edge of the reference holes from a counterbalance. The precision of the radial position measurement is about +/−0.001 cm, which is sufficient for most quantitative analysis. The temperature of the rotor is monitored by a radiometer and regulated by thermoelectric modules. The temperature fluctuation of the refrigeration usually does not exceed 1ºC, and the corresponding rotor and sample temperature fluctuation is less than 0.02ºC upon reaching thermal equilibrium.[20]

The protein concentrations during a centrifugation run are measured by either interference or absorption optical systems in XLI. Both detection methods offer their unique advantages. The interference optical system measures the protein concentration based on changes in refractive index. It provides rapid, high precision data acquisition for samples at a broad range of concentrations.[9,21] In addition, the interference system can be applied to molecular species that do not contain chromophores, which provide a significant absorbance in the UV-visible range. In contrast, the absorption optical system measures protein concentration based upon the fact that many macromolecular species, such as protein and DNA, contain chromophores that absorb incident light in the UV range. The concentration of macromolecules is calculated according to Beer's law. The absorption optical system offers very high sensitivity and can discriminate between molecules with different chromophores.

The absorption optical system uses a Xenon flash lamp, providing a usable wavelength range over 190 to 800 nm. The light goes through a toroidal diffraction grating monochromator to produce incident light at a single wavelength. The accuracy of wavelength is calibrated with the intensity profile of incident light and verified by a wavelength reference cell that contains a holmium oxide filter. The typical accuracy of wavelength is approximately ±2 nm. The light source for the interference optical system uses a 30-mW, 660-nm laser. The interference patterns are recorded digitally on a charge-coupled device (CCD) camera and then converted to a graphical representation of fringe displacement as a function of radial position.

A small drift of wavelength in the absorption optical system can often happen during the radial scans when the wavelength setting is changed. For example, the wavelength can drift slightly when repetitive scans at more than 1 wavelength are used. This drift is usually less than 2 nm. In a typical sedimentation velocity run, the scans are only conducted at a single wavelength, therefore the wavelength drift normally should be minimized within all the scans. However, wavelength drift can become a significant problem in some of the older X-linked agammaglobulinemia (XLA) instruments, particularly when the data are collected at a low wavelength, where the absorbance is on the shoulder of the spectrum. Figure 2A shows an example of wavelength drift problem that we observed during a sedimentation

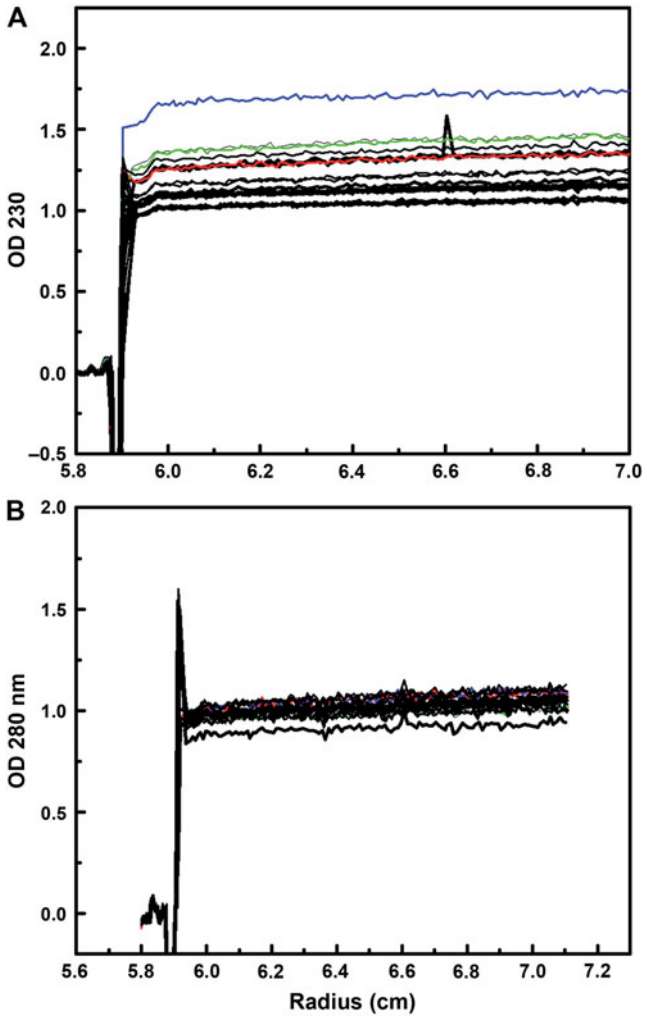

Figure 2. Sedimentation velocity experiment of a tryptophan solution at 40 μg/mL. The data were collected at 3000 rpm and 20ºC at a wavelength of 230 nm (A) or 280 nm (B).

velocity experiment. In this analysis, we used L-tryptophan as a reporter chemical agent; because it has a strong absorbance at 230 and 280 nm, and due to its small size, it does not undergo appreciable sedimentation at 3000 rpm. As shown in Figure 2A, the UV absorbance at the plateaus of the L-tryptophan sample at 40 μg/mL measured at 230 nm during centrifugation at 3000 rpm clearly shows a significant drift of absorbance between different scans. The drift appears to be more noticeable and frequent with the early scans. The magnitude of change is much less noticeable at 280 nm, where the tryptophan has a maximum absorbance peak (Figure 2B). In the worst situation seen so far, there is almost a 50% change on absorbance; this only corresponds to a 1 to 2 nm drift of wavelength. The systematic error resulting from wavelength drift cannot be easily corrected using currently available software, and it can have significant effect on measuring low levels of protein aggregates. As shown in Figure 3, for a simulated irreversible monomer and dimer system (99% monomer, 1% dimer), where 10% of scans have a 5% change on absorbance, analysis of these data by SEDFIT results in variability in the estimate of aggregate species. Not only is the sedimentation coefficient of the dimer changed but also the level of soluble dimer is underestimated. Of interest, a small deviation on wavelength does not appear to have a significant effect on the main peak. Although the percentage of the main peak does change slightly, the sedimentation coefficient of this peak is essentially the same as that expected for the monomer.

Small drifts on wavelength are common in sedimentation equilibrium runs, where multiple wavelengths are used.

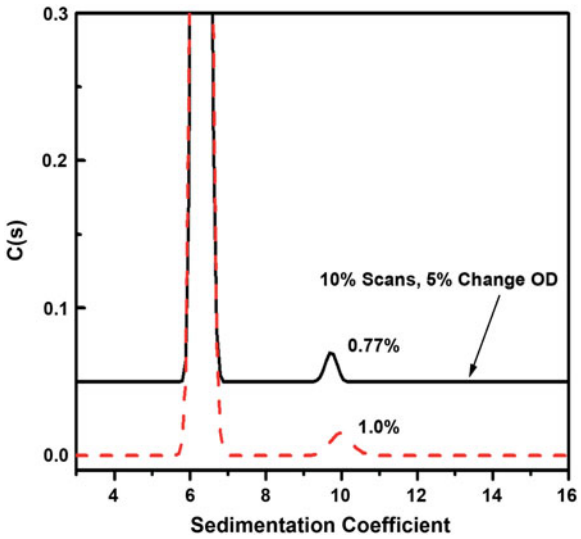

Figure 3. Simulation of a sedimentation velocity study of a noninteracting discrete species model. The total absorbance is set at 1 optical density (OD). The model contains 99% of monomer and 1% of dimer. The molecular weights are 150 kd for the monomer and 300 kd for the dimer. The solid line represents data containing 0% of scans with 5% changes on OD, and the dashed line represents data containing no systematic error.

This drift usually is less than 2 nm. The small changes of intensity can be corrected using nonlinear least squares fitting program. For sedimentation velocity experiment, it is recommended that the continuous scan be conducted at a single wavelength. In addition, the wavelength for scans should be set at or near the peak of UV absorbance, rather than its shoulder, to minimize the effect of wavelength drift on the concentration determination. The wavelength drift problem during the sedimentation velocity experiment can be easily detected using an L-tryptophan solution, since the absorbance changes quite significantly around the 230 nm region. As discussed above, a few nm changes in wavelength at 230 nm can result in almost a 50% change on the absorbance level.

Effects of Systematic and Random Noise

The data collected from analytical ultracentrifuge study can have both systematic and random noises. The signal:noise ratio is an important parameter that will govern the sensitivity and accuracy of the measurement. There are 2 kinds of basic systematic noises from an AUC experiment. The first is the background noise; also know as time-invariant noise (TI). This noise often results from dust or scratches on the window, or the imperfection in some of the optical components of the interference optics. The second is radial invariant noise (RI) that can change on each scan. Traditionally, RI noise was corrected by alignment of the scans over a range of radial values, and TI noise was eliminated by analyzing pairwise differences of scans. This approach has been used in the dc/dt programs.[18,22] The other approach uses a least squares fitting method to correct both RI and TI noises. This approach is used in the SEDFIT program.[12] In addition to systemic noise, the data from AUC study also contain small, but noticeable, levels of random noise because of intrinsic photometric noise of the optical system. This baseline noise also can have a significant effect on the sensitivity of measurement, particularly when aggregate level is low.

Figure 4 shows the mathematical simulation data from a noninteracting monomer and dimer model. The samples contain 99% of monomer and 1% of dimer. The random noise from 1% to 5% of total signal was applied to the data. As noise level increased, the signal-to-noise ratio decreased, and not only the amounts of aggregate but also the sedimentation coefficient of aggregate determined by SEDFIT analysis deviated from the theoretical values. It is clear from this study that the random noise level will have an effect on the detection limit for protein aggregates. For a typical absorbance optical system, the baseline noise level is around 0.006 optical density (OD). This corresponds to ~0.6% of total sample signal if the samples were prepared at 1 OD. Figure 5 shows results in which the model contains either 0.1% or 0.5% dimer, respectively. Although SEDFIT is

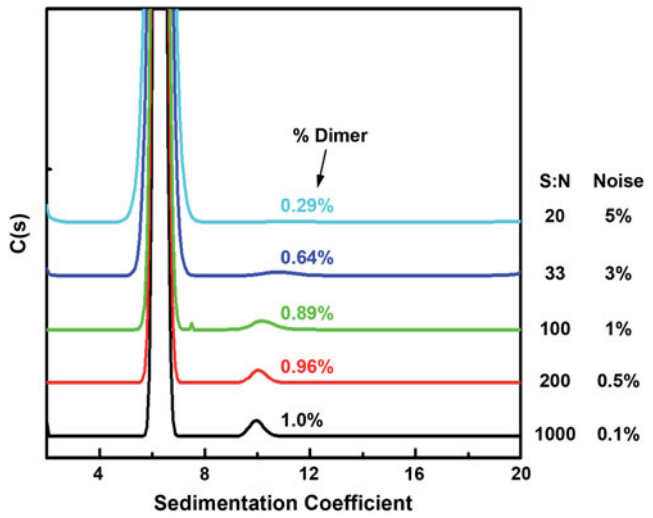

Figure 4. Simulation of a sedimentation velocity study of a noninteracting discrete species model. The total absorbance is set at 1 optical density (OD). The model contains 99% of monomer and 1% of dimer. The signal:noise (S:N) ratio was increased from 20 to 1000.

capable of resolving dimer in both scenarios, the accuracy of the measurement is much worse in the sample containing 0.1% aggregate than in the 0.5% aggregate.

Effects of Meniscus Position and Frictional Ratio

The meniscus position in sedimentation velocity experiment often occurs as a sharp absorbance peak using absorbance optical system but is less noticeable using interference optical system. The meniscus position corresponding to the starting position of sedimentation is a critical parameter for SEDFIT analysis. Although the meniscus position can be obtained by least squares fitting, it does not always return to the peak positions. As illustrated below, a slight shift of meniscus from the peak position can have a significant effect on the quantitative analysis of small amounts of protein aggregates.

Figure 6 shows mathematical simulation data of a noninteracting monomer and dimer system. The model contains 99% monomer and 1% dimer with a monomer molecular weight at 150 kd. The theoretical meniscus position was set at 6.2 cm. The data were analyzed by SEDFIT with the meniscus fixed at the different positions. As the meniscus position shifted away from the theoretical set position, the size distribution of aggregates changed accordingly. The c(s) analysis yielded aggregates with very different size than the dimer form. In addition, it produced artificial fragment and aggregate peaks that did not exist in the original model. Similar results were also obtained from a sedimentation velocity analysis of a monoclonal antibody preparation (Figure 7). This sample contains ~1% of soluble aggregates as determined by the SEC method. When the meniscus position was set at near the middle point of the meniscus peak, the percentage of aggregate from the c(s) plot was consistent with that obtained from SEC. However, if the meniscus position was shifted to the right side of the middle point, the size distribution of aggregates increased. Likewise, as the meniscus position shifted to the left side of the midpoint, the size distribution of aggregates decreased, while fragment peak increased. The slight shift of the meniscus position often has limited effect on the goodness of fit but clearly is essential for measuring small amounts of aggregates.

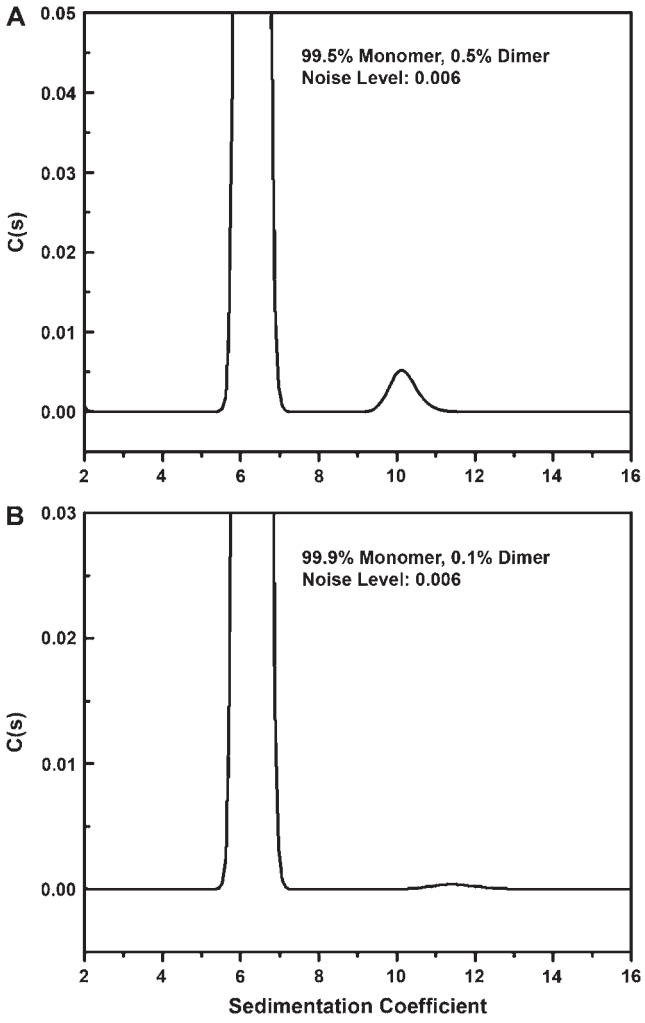

Figure 5. Simulation of a sedimentation velocity study of a noninteracting discrete species model. The total absorbance is set at 1 optical density (OD). The model contains either (A) 99.5% monomer, 0.5% dimer; or (B) 99.9% monomer, 0.1% dimer.

The ratio of frictional coefficient is another key parameter for aggregate quantitation by SEDFIT analyses. In SEDFIT analysis using the c(s) method, it is assumed that all species have the similar hydrodynamic shape. For heterogeneous samples, the fractional ratio corresponds to the weight average value of all species. This assumption is not always valid with complex systems containing multiple species with different hydrodynamic shapes. Table 1 shows the example of

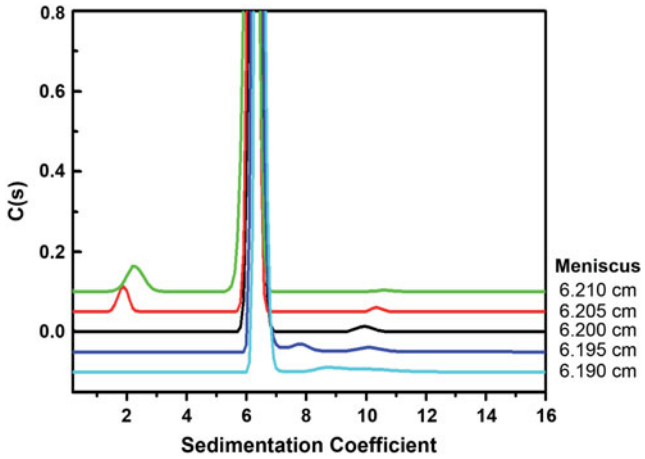

Figure 6. Simulation of a sedimentation velocity study of a noninteracting discrete species model. The total absorbance is set at 1 optical density (OD). The model contains 99% monomer and 1% dimer. The C(s) plot was generated by fitting the data with different prespecified meniscus positions.

SEDFIT analysis of an intact IgG1 and antibody Fab fragment mixture when these components were mixed at different weight fractions. The mixed samples were then analyzed by SEC and sedimentation velocity analyses. The frictional ratio of Fab was determined as 1.27, while the value for IgG1 was ~1.53. As shown in Table 1, the percentage of Fab and IgG1 in the mixed samples determined by SEC was exactly as expected. However, the result from c(s) analysis using continuous distribution was clearly different from the theoretical value. It underestimated the Fab level but overestimated the IgG1 level. One way to overcome this problem is to use the biomodal model in c(s) analysis. This model can handle 2 species with very different shape and conformation. The frictional ratio of each species can be resolved simultaneously. As shown in Table 1, the percentage of aggregates determined by c(s) plot using the biomodal model was exactly the same as what we expected.

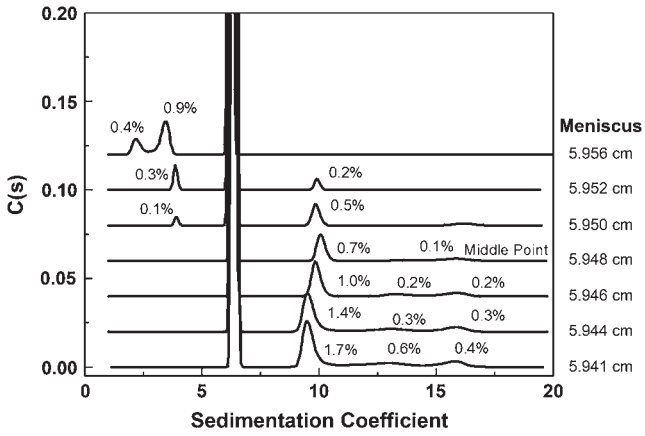

Figure 7. Sedimentation velocity experiment of a mAb. The sample contains ~1% of soluble aggregates. The C(s) plot was generated by fitting the data with different fixed meniscus positions.

Although many of the folded protein monomer and aggregates have a similar frictional ratio, and quantitation of monomer and aggregate species can be determined using c(s) plot with reasonable confidence, our results suggest that better quantitation of a sample containing multiple components can be obtained when the frictional ratio of each individual species is determined and verified by an independent measurement. Although this may involve using orthogonal methods to purify components, the results may significantly improve the precision and accuracy of a quantitative analysis for complex systems.

Considerations for Improving Experimental Conditions and Data Analysis

As we have discussed, AUC is an extremely powerful method for characterizing proteins in solution. To increase its sensitivity, it requires a careful maintenance of instrumentation and additional steps to minimize both systematic and random noise. The wavelength, radial position, and rotor temperature should be checked and calibrated regularly using reference or internal standards to ensure the instrument is performing properly. For experimental setup, it is important to avoid the use of thermodynamically nonideal conditions, such as low salt and high protein concentrations, since the current version of SEDFIT program is not designed to handle non-ideal systems. The wavelength should be selected at an absorbance peak at which the absorbance signal does not change greatly as a function of small wavelength drift. This method can minimize the potential wavelength drift problem. The numbers of scans are also important for quantitative analysis. In general, the more scans used for data analysis, the more reliable and reproducible the result will be. For the absorbance optical system, the increase of scan number can be achieved by lowering the temperature, using larger step size, or simply running a single cell in an analysis. For sedimentation velocity data analysis with SEDFIT, it is important to assess if the model fits well with the data in terms of root-mean-square deviation (RMSD) and residual distribution. The bit map plot is also very useful to identify the systematic deviation of the fitted curve from the data. Although the meniscus can be obtained by fitting the data directly, it should be compared with the meniscus peak position to ensure it is still within the expected range. In many cases, a constant frictional ratio assumption used in SEDFIT is valid but should be verified if the aggregates can be separated and collected for further characterization. For a system where there are significant differences in frictional ratio between individual species, the c(s) analysis using a weight average value can produce large errors on quantitation. If the system is bimodal, this problem can be overcome by fitting the frictional ratios for each individual species separately. For better quantitative analysis, it is also important to run the same sample at multiple times, as this

Table 1. Summary of Fab-IgG1 Mixture Determined by SEC and SEDFIT*

Sample Mixture	SEC		SEDFIT (weight average)		SEDFIT (biomodal)	
	Fab	IgG1	Fab	IgG1	Fab	IgG1
5% Fab, 95% IgG1	5%	95%	4%	96%	5%	95%
10% Fab, 90% IgG1	10%	90%	8%	92%	10%	90%
20% Fab, 80% IgG1	20%	80%	18%	82%	20%	80%

*SEC indicates size exclusion chromatography.

will provide statistical information of the results. Finally, as is true of any biophysical methods, it is important to remember that there are always limitations and assumptions that may not fit to every situation. The results for AUC should also be verified using other orthogonal approaches.

Field Flow Fractionation

Background

Field flow fractionation (FFF) is a flow-based separation methodology. It separates the macromolecules based on their differences of diffusion coefficients.[6] The retention and separation of samples are controlled by an external field, perpendicular to channel flow. There are several different fields that can be used for separation of protein with FFF technology. These include flow, sedimentation, electrical, and thermal FFF.[7] Among all these methods, flow FFF technology has been widely used and is most suitable for separation of protein aggregates.

During flow FFF operation, samples were injected into a thin and elongated flow chamber, known as a channel. A semipermeable membrane, permeable to the elution buffer but not to the samples, is used as the accumulation wall to prevent samples from exiting the channel via the cross-flow outlet. The samples are first focused into a narrow band by 2 opposite flows, and then eluted with a continuation of flow into a detector. In the normal elution mode, the smaller molecules have larger diffusion coefficients and tend to diffuse and concentrate closer to the center of laminar flow where the flow rate along the length of channel is faster, resulting in an earlier elution than for the larger molecules. Unlike conventional chromatography methods, there is no stationary phase involved. All separation happens in a single phase, thereby eliminating the potential problems caused by matrix-protein interaction. Although the membrane-protein interaction still exists in FFF channel, particularly at high cross-flow conditions, most of these problems can be minimized by selecting low absorption membranes, such as regenerated cellulose. For better separation, the membrane must be thin, smooth, flat, and free of creases.

Reproducibility

Figure 8 shows the results of FFF studies with 2 mAb products. The sample was injected in duplicate or triplicate. The cross-flow was set at 3 mL/min and channel flow at 0.5 mL/min. The main peak eluted at ~10 minutes corresponds to a monomer peak. The determined elution time and total peak

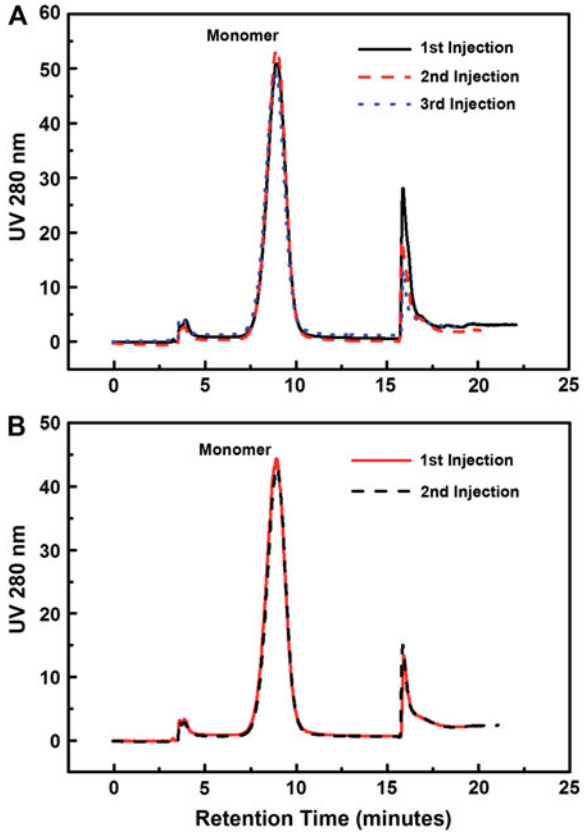

Figure 8. FFF analysis of 2 mAbs. Each mAb was injected in triplicate (A) or duplicate (B). The samples were eluted in the phosphate buffered solution (PBS) at cross-flow rate of 3 mg/mL.

area for each mAb monomer is quite consistent for the repeat injections. The second peak, which is often referred to as the large aggregate or particle peak, was eluted when cross-flow approached zero. There are slight differences between each injection. This is probably owing to the changes in the flow rate while the flow ramps down. The total "aggregate peak" is larger than what we usually expect for a highly purified mAb. Further study of the molecular weight with an on-line light scattering detector indicated that the second peak was mainly the monomer form. This result suggests that there is significant nonspecific absorption of protein on the membrane during the run while the cross-flow is high. The nonspecific absorption of protein on FFF membrane appears to be quite common and happens on all the runs and membranes we have tested so far. This problem is mostly controlled by reducing the cross-flow.

Analysis of Size Distribution of Protein

The size distribution of a degraded mAb was evaluated using the FFF method. Figure 9 shows the result of the FFF study of an mAb stored at 30ºC and −70ºC. The cross-flow was set at 6 mL/min, which has produced a separation for smaller size fragments with a high resolution. The 2 fragments have molecular weights of 50 and 100 kd, respectively, which is consistent with the size of a Fab and 1-armed antibody. The high cross-flow and low channel-flow combination increases the power of separation for small size fragments. The resolution is better than can be achieved with typical SEC chromatography.

The FFF method can also be used to separate soluble protein aggregates. In normal separation mode, the larger size protein aggregates will be closer to the membrane owing to the relatively small values of their diffusion coefficient. The aggregates tend to stay closer to ultrafiltration (UF) membrane, and therefore will be eluted after the monomer. For aggregate separation, the cross-flow is always set at relatively low value to prevent the aggregate from getting too close to the membrane. As shown in Figure 10A, at a cross-flow of 3 mL/min, the aggregates were clearly separated from its monomer. The aggregate peaks resolved in FFF appear to have a little broader distribution; therefore the resolution is not as good as SEC and sedimentation velocity. In spite of these drawbacks, FFF is still a very useful method, particularly for monitoring the large and insoluble aggregates. As shown in Figure 10B, the larger and insoluble aggregates that are not detectable due to the filtration of matrix, can be easily detected in FFF. This peak can be further characterized using an on-line light scattering method.

Summary of FFF Technology

FFF method is best suited for analyzing larger aggregates that can be easily filtered by a SEC column matrix or sediment under centrifugal field. The method provided good separation for antibody fragments, but limited separation

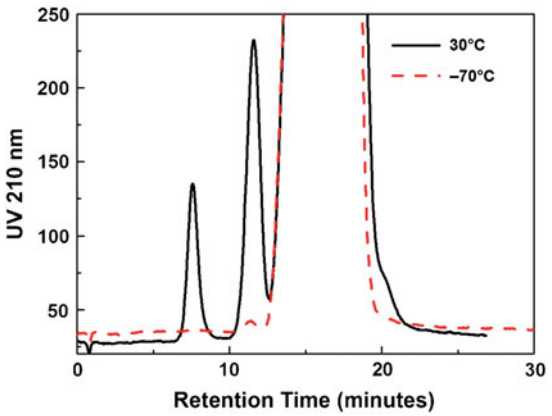

Figure 9. FFF analysis of mAb fragments. The samples were stored at 30ºC (—) and −70ºC (—). The samples were eluted at a cross-flow rate of 6 mL/min and a channel flow rate of 0.2 mL/min.

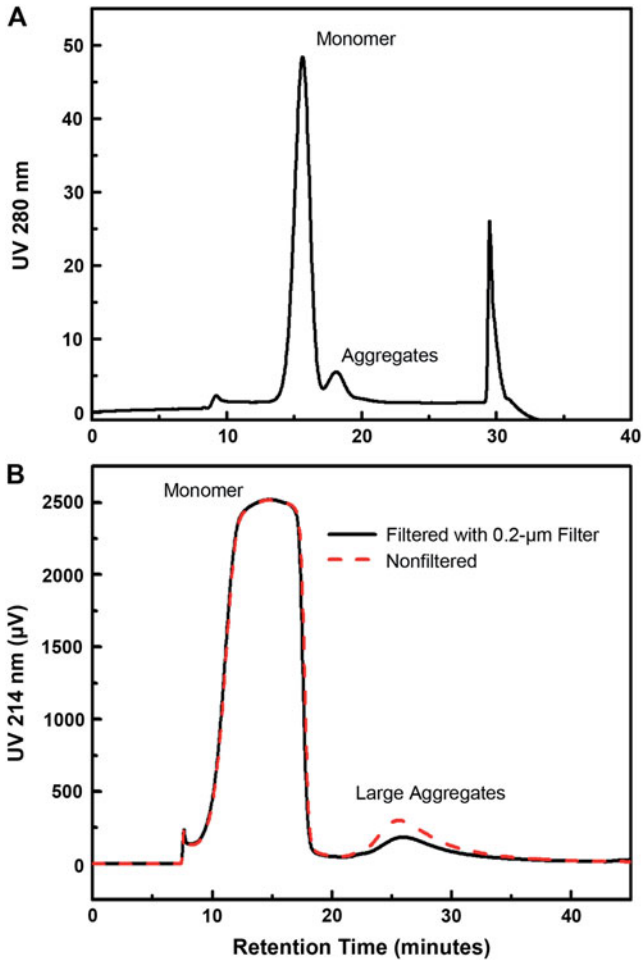

Figure 10. FFF analysis of mAb aggregates. The soluble aggregates (A) were eluted at a cross-flow of 3 mL/min, and large or insoluble aggregates (B) were eluted at a cross-flow rate of 0.5 mL/min.

for soluble antibody aggregates. The method has a broad dynamic range and can be used to study protein that are fragile and shear sensitive.

CONCLUSIONS

Both AUC and FFF are powerful approaches for measuring and characterizing protein aggregates. With appropriate procedure, they can provide complementary information, and are well suited to serve as orthogonal methods to SEC-HPLC.

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REFERENCES

1. Schellekens H. Factors influencing the immunogenicity of therapeutic proteins. *Nephrol Dial Transplant*. 2005;20:vi3-vi9.

2. Hermeling S, Crommelin DJ, Schellekens H, Jiskoot W. Structure-immunogenicity relationships of therapeutic proteins. *Pharm Res*. 2004;21:897-903.

3. Robbins DC, Hirshman M, Wardzala LJ, Horton ES. High-molecular-weight aggregates of therapeutic insulin: in vitro measurements of receptor binding and bioactivity. *Diabetes*. 1988;37:56-59.

4. Maislos M, Bialer M, Mead PM, Robbins DC. Pharmacokinetic model of circulating covalent aggregates of insulin. *Diabetes*. 1988;37:1059-1063.

5. Lebowitz J, Lewis MS, Schuck P. Modern analytical ultracentrifugation in protein science: a tutorial review. *Protein Sci*. 2002;11:2067-2079.

6. Giddings JC. Field-flow fractionation: analysis of macromolecular, colloidal, and particulate materials. *Science*. 1993;260:1456-1465.

7. Calvin GJ. *The Field-Flow Fractionation Family: Underlying Principles.* New York, NY: John Wiley & Sons; 2000.

8. Reschiglian P, Zattoni A, Roda B, Michelini E, Roda A. Field-flow fractionation and biotechnology. *Trends Biotechnol*. 2005;23:475-483.

9. Laue TM, 3rd, Stafford WF, 3rd. Modern applications of analytical ultracentrifugation. *Annu Rev Biophys Biomol Struct*. 1999;28:75-100.

10. Schuster TM, Toedt JM. New revolutions in the evolution of analytical ultracentrifugation. *Curr Opin Struct Biol*. 1996;6:650-658.

11. Stafford WF, 3rd. Boundary analysis in sedimentation velocity experiments. *Methods Enzymol*. 1994;240:478-501.

12. Schuck P. Size-distribution analysis of macromolecules by sedimentation velocity ultracentrifugation and Lamm equation modeling. *Biophys J*. 2000;78:1606-1619.

13. Hansen JC, Lebowitz J, Demeler B. Analytical ultracentrifugation of complex macromolecular systems. *Biochemistry*. 1994;33:13155-13163.

14. Dam J, Velikovsky CA, Mariuzza RA, Urbanke C, Schuck P. Sedimentation velocity analysis of heterogeneous protein-protein interactions: Lamm equation modeling and sedimentation coefficient distributions c(s). *Biophys J*. 2005;89:619-634.

15. Dam J, Schuck P. Sedimentation velocity analysis of heterogeneous protein-protein interactions: sedimentation coefficient distributions c(s) and asymptotic boundary profiles from Gilbert-Jenkins theory. *Biophys J*. 2005;89:651-666.

16. Correia JJ. Analysis of weight average sedimentation velocity data. *Methods Enzymol*. 2000;321:81-100.

17. Demeler B, Saber H, Hansen JC. Identification and interpretation of complexity in sedimentation velocity boundaries. *Biophys J*. 1997;72:397-407.

18. Stafford WF, 3rd. Boundary analysis in sedimentation transport experiments: a procedure for obtaining sedimentation coefficient distributions using the time derivative of the concentration profile. *Anal Biochem*. 1992;203:295-301.

19. Brookes E, Demeler B. Genetic Algorithm Optimization for Obtaining Accurate Molecular Weight Distributions from Sedimentation Velocity Experiments. In: Wandrey C, Cölfen H, eds. *Progress in Colloid and Polymer Science-Analytical Ultracentrifugation.* 8th ed. Heidelberg, Germany: Springer-Verlag; 2006:33-40.

20. Robert G. *The Optima XL-A: A New Analytical Ultracentrifuge With a Novel Precision Absorption Optical System.* London, UK: The Royal Society of Chemistry; 1992.

21. Allen F. The XL-I analytical ultracentrifuge with Rayleigh interference optics. *Eur Biophys J*. 1997;35:307-310.

22. Philo JS. An improved function for fitting sedimentation velocity data for low- molecular-weight solutes. *Biophys J*. 1997;72:435-444.